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Insertion of a foreign sequence on capsid surface loops of human papillomavirus type 16 virus-like particles reduces their capacity to induce neutralizing antibodies and delineates a conformational neutralizing epitope

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Abstract

The aims of this study were to generate chimeric human papillomavirus (HPV)-16 L1 virus-like particles (VLPs) in order to identify immunogenic domains and conformational neutralizing epitopes, and to characterize the regions where a foreign epitope could be introduced. We hypothesized that these regions could be on L1 protein loops since they are exposed on the surface of VLPs. The aims of this study were achieved by mutating HPV-16 L1 proteins. Six amino acids encoding for the epitope 78–83 (DPASRE) of the hepatitis B core (HBc) antigen were introduced within the different loops of the L1 protein at positions 56/57, 140/141, 179/180, 266/267, 283/284 or 352/353. All these chimeric L1 proteins were capable of self-assembly into VLPs. The antigenicity and immunogenicity of some of these VLPs were reduced compared to the levels observed with wild-type VLPs. All were nevertheless able to induce neutralizing antibodies. VLPs with insertion at position 266/267 induced lower levels of neutralizing antibodies, suggesting the involvement of residues situated on FG loop in L1 neutralizing epitopes. All the chimeric L1 proteins except the one with insertion at position 56/57 were also able to induce anti-HBc antibodies, thus suggesting exposure of the HBc epitope on the VLP surface. Taken together, our findings indicate the possibility of designing HPV-derived vectors that are less immunogenic and suggest positions for insertion of defined immune epitopes or cell ligands into L1 protein to be exposed on the surface of VLPs.

Keywords: HPV; Hepatitis B core; Immunogenicity; Antigenicity

Introduction

Over 100 human papillomaviruses (HPV) have been identified to date (Stoler, 2000). They induce benign epidermal and mucosal papillomas. In addition, the development of cervical cancer, the second greatest cause of cancer deaths in women worldwide (Ferlay et al., 1998), is strongly associated with genital infection by specific types, such as HPV-16, 18, 31, 33, 39, 45, 52, and 58 (Munoz, 2000; Walboomers et al., 1999). Papillomaviruses are nonenveloped viruses with an 8-kb double-stranded circular DNA for the genome, encapsidated in a structure consisting of 72 capsomers composed of L1 and L2 proteins, the major and minor capsid proteins, respectively (Baker et al., 1991; Trus et al., 1997). The major capsid protein of HPV can self-assemble into virus-like particles which have the size and shape of virions (Kirnbauer et al., 1993; Le Cann et al., 1994; Rose et al., 1993; Rossi et al., 2000).

Both linear and conformational epitopes have been identified on the surface of HPV L1 VLPs (Christensen et al., 1996). It is now well established that conformational

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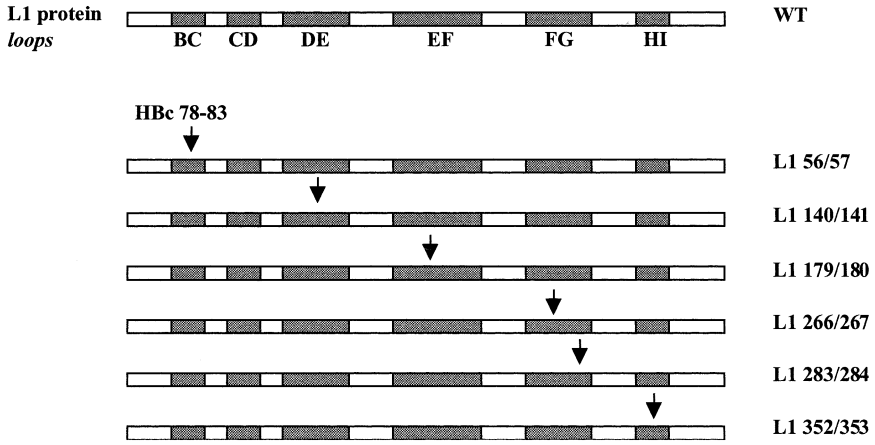

Fig. 1. Schematic representation of HBc/HPV-16 L1 chimeric proteins.

epitopes are responsible for neutralizing antibody production (Christensen et al., 1994b; Giroglou et al., 2001; Rose et al., 1994; White et al., 1998, 1999). The tertiary structure of the L1 protein of HPV-16 is that of a jellyroll β sandwich formed by the conserved sequences with additional hypervariable loops (Chen et al., 2000). The crystal structure of HPV-16 L1 pentamers shows that the hypervariable loop domains extending toward the outer surface of the capsid (Chen et al., 2000). Mutagenesis of the hypervariable loop domains has previously revealed the binding sites of several neutralizing monoclonal antibodies generated against HPV-11, including residues 120–140, 131–132, 246, and 346 (Ludmerer et al., 1996, 1997), or against HPV-6, including residues 49–54 and 169–178 (McClements et al., 2001). In addition, L1 residues 50, 266, and 282 of HPV-16 have been implicated in the binding of neutralizing antibodies (Roden et al., 1997; White et al., 1999). Slupetzky et al. (2001) recently confirmed that regions around residues 282–286 and 351–355 contribute to BPV-1 L1 neutralizing epitopes.

The aims of this study were to generate chimeric HPV-16 L1 VLPs (HPV cVLPs) in order to identify immunogenic domains and conformational neutralizing epitopes. We introduced a foreign sequence of six amino acids (aa) encoding the epitope 78–83 (DPASRE) of the hepatitis B core (HBc) antigen (Conway et al., 1998; Sallberg et al., 1991) within the different hypervariable loops of the L1 protein. The chimeric L1 proteins generated were tested for assembly into VLPs, for antigenicity and immunogenicity, including their capacity to induce neutralizing antibodies, and for their capacity to present the foreign epitope.

Results

Insertion of the HBc78–83 epitope into surface loops of HPV-16 L1 protein does not interfere with capsid assembly

To obtain the chimeric HBc/HPV-16 L1 proteins, the HBc epitope (DPASRE) was inserted between residues 56/57, 140/141, 179/180, 266/267, 283/284, or 352/353 of the L1 protein, corresponding to insertion in loops BC, DE, EF, FG, and HI, respectively (Fig. 1). All chimeric proteins were able to self-assemble into VLPs, which have a similar shape to VLPs composed of wild-type (WT) L1 protein (Fig. 2). Chimeric VLPs with insertion at positions 140/141, 179/180, 352/353, and 283/284 are the same size as wild-type VLPs (50 nm). HPV-16 L1 mutant 56/57 produced VLPs of 50 nm but also larger VLPs of 65 nm, and mutant 266/267 with insertion in the FG loop produced VLPs of 50 nm but also smaller VLPs of around 40 nm.

For the subsequent experiments, VLP preparations were standardized according to their protein content, which is consistent with their reactivity with CamVir-1 MAb when denatured (data not shown).

Insertions into the FG loop of HPV-16 L1 protein reduced VLP antigenicity

The antigenicity of chimeric VLPs was investigated using sera from mice immunized with wild-type HPV-16 VLPs. The reactivity of these sera against chimeric VLPs with insertion at position 140/141 was similar to that observed with wild-type HPV-16 VLPs (Table 1). In contrast, reactivity was reduced when L1 was mutated at other positions. A dramatic decrease was observed when the HBc78–83 epitope was inserted into the FG loop at positions 266/267 or 283/284 (Table 1).

The antigenicity of chimeric HPV-16 VLPs was also analyzed using four anti-HPV-16 L1 Mabs, including CamVir-1 directed against a linear epitope (amino acids 204–210) and H16.U4, H16.V5, and H16.E70 directed against conformational epitopes. The reactivity of CamVir-1 and H16.U4 MAbs toward chimeric and wild-type HPV-16 VLPs was similar (Table 1). Binding of H16.V5 and H16.E70 MAbs to chimeric VLPs, with HBc epitope insertion at position 56/57, 140/141, 179/180, or 352/353, was identical to binding on wild-type HPV-16 VLPs. In contrast, binding of these MAbs to the other two chimeric VLPs with

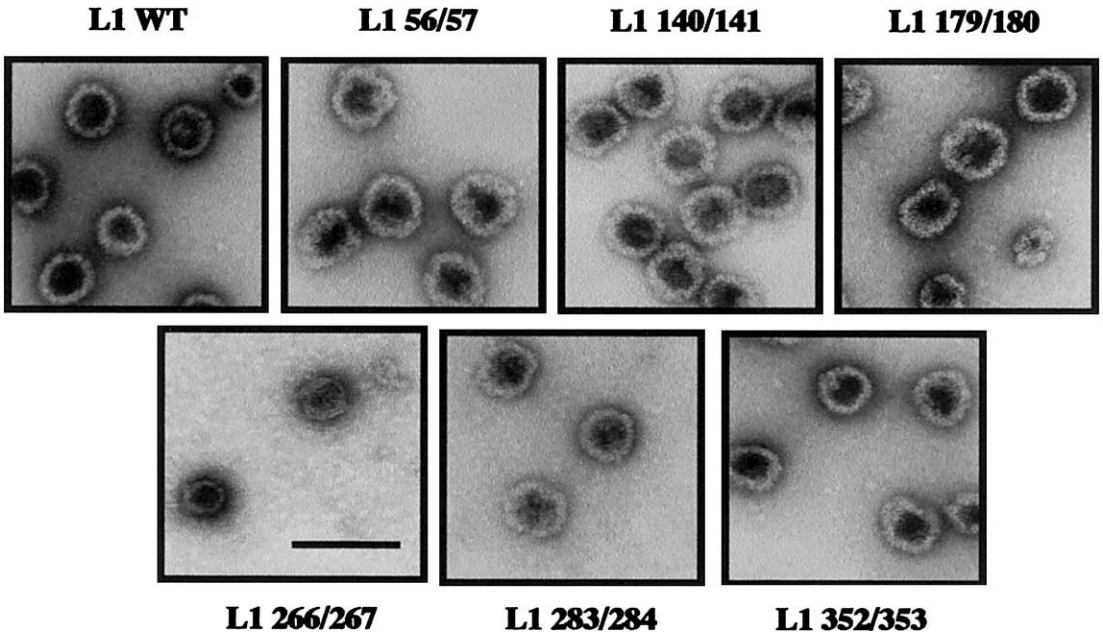

Fig. 2. Electron micrographs of HPV VLPs composed of wild-type HPV-16 L1 protein or chimeric HBc/HPV-16 L1 protein with insertion at position 56/57, 140/141, 179/180, 266/267, 283/284, or 352/353 are shown. VLPs were purified from nuclear extracts of Sf21 cells infected by recombinant baculovirus encoding wild-type HPV-16 L1 or chimeric HBc/HPV-16 L1 proteins and then observed by transmission electron microscopy at a magnification of 1/50,000 (the bar represents 100 nm).

insertion at position 266/267 or 283/284 (HPV-16 VLPs mutated on FG loop) was considerably reduced.

Insertions into the FG loop of HPV-16 L1 protein reduced the immunogenicity of the corresponding VLPs

The immunogenicity of chimeric VLPs was investigated by testing the reactivity of the sera of four mice immunized with wild-type or chimeric VLPs. Figure 3 shows that reactivity to HPV-16 VLPs of sera from mice immunized with L1 proteins with insertion at position 140/141 or 179/180 was similar to that observed with sera from mice immunized with wild-type L1 VLPs. In contrast, sera from mice immunized with the other chimeric VLPs exhibited lower reactivity. The reduction was more pronounced with sera from mice immunized with VLPs composed of L1 proteins with insertion of the HBc epitope at position 266/267 (Fig. 3) and was at a lower level for insertion at position 56/57, 283/284, or 352/353. Low reactivity of sera from mice immunized with VLPs with HBc insertion at position 266/267 was also observed when tested against autologous L1 226/267 VLPs. In addition, similar to the reactivity observed with wild-type VLPs, a less pronounced reduction in reactivity was observed with sera from mice immunized with VLPs with insertion at positions 56/57, 283/284, and 352/353 when tested with each of the autologous VLPs, respectively. Induction of HPV-16 neutralizing antibodies was also estimated by testing the capacity of sera from immunized mice to interfere with gene transfer by HPV-16 pseudovirions (Table 2). Neutralizing antibodies were de-

Table 1
Anti-VLP titers of monoclonal and polyclonal anti-L1 antibodies when tested against WT HPV-16 L1 VLPs and chimeric HBc/HPV-16 L1 VLPs

L1 VLPs	Anti-VLP antibody titers				
	Monoclonal				Polyclonal
	Cam Vir-1	H16.U4	H16.V5	H16.E70	anti-VLPs
WT	$3.2\ 10^3$	$7.1\ 10^4$	$7.8\ 10^6$	$1.4\ 10^5$	$1.2\ 10^4$
L1 56/57	$3.2\ 10^3$	$1.4\ 10^5$	$7.8\ 10^6$	$1.4\ 10^5$	$1.6\ 10^3$
L1 140/141	$3.2\ 10^3$	$1.4\ 10^5$	$7.8\ 10^6$	$7.1\ 10^4$	$2.5\ 10^4$
L1 179/180	$3.2\ 10^3$	$1.4\ 10^5$	$7.8\ 10^6$	$1.4\ 10^5$	$3.2\ 10^3$
L1 266/267	$1.3\ 10^4$	$1.4\ 10^5$	$1.0\ 10^2$	$4.0\ 10^2$	$4.0\ 10^2$
L1 283/284	$6.4\ 10^3$	$7.1\ 10^4$	$1.0\ 10^3$	$<2.0\ 10^2$	$2.0\ 10^2$
L1 352/353	$1.3\ 10^4$	$2.8\ 10^5$	$7.8\ 10^6$	$1.4\ 10^5$	$3.2\ 10^3$

Note. Titers were defined as the highest serial dilution which yielded an OD value greater than 0.200.

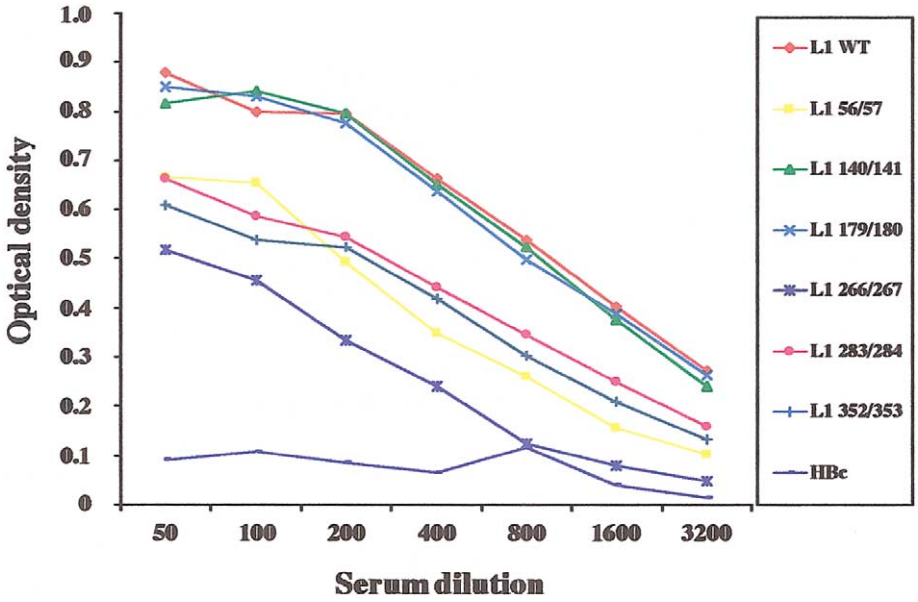

Fig. 3. The immunogenicity of chimeric VLPs: detection of anti-HPV-16 VLP antibodies in serum of mice immunized with WT and chimeric VLPs is shown. Anti-VLP antibodies were obtained by subcutaneous immunization of four mice with purified WT or chimeric VLPs at days 0, 6, 14, and 28. Blood samples were collected 11 days after the last injection and anti-VLP reactivity was investigated by ELISA using WT VLPs as antigen. OD values reported are the mean of the OD values observed in each of the four mice.

tected in all mice immunized with wild-type HPV-16 VLPs or chimeric VLPs and in none of those immunized with HBc VLPs. A geometric mean titer of 2393 was observed with wild-type VLPs, whereas it was only 237 in mice immunized with mutant 266/267. A much lower reduction in the geometric mean titer of neutralizing antibodies was observed with other chimeric VLPs, with titers ranging from 673 to 1131.

Table 2
Immunogenicity of chimeric HBc/HPV-16 L1 VLPs: number of mice with neutralizing antibodies against HPV VLPs (1/50) and antibodies against HBc VLPs (1/100)

VLPs	HPV-neutralizing antibodies		Anti-HBc	
	Positive/tested	GMT[a]	Positive/tested	GMT[a]
L1 WT	4/4	2393	0/4	<50
L1 56/57	4/4	673	0/4	<50
L1 140/141	4/4	673	2/4	84
L1 179/180	4/4	800	2/4	100
L1 266/267	4/4	237	4/4	237
L1 283/284	4/4	1131	2/4	100
L1 352/353	3/3	800	3/3	79
HBc	0/4	<50	4/4	>3200

Note. HPV neutralizing antibody titers were defined as the highest serial dilution which yielded more than 80% neutralization of the luciferase expression obtained using HPV-16 pseudovirions. Anti-HBc ELISA titers were defined as the highest serial dilution which yielded an OD value greater than 0.150.

[a] GMT, geometric mean titers.

The HBc78–83 epitope inserted in HPV-16 L1 loops is exposed on the surface of the VLPs

To evaluate whether the inserted HBc sequence was present on the surface of the particles, the chimeric HPV-16 L1 proteins were investigated for their capacity to induce anti-HBc antibodies. Sera of four mice immunized with HBc or chimeric HBc/HPV-16 L1 VLPs were analyzed by ELISA using HBc VLPs. The results (Table 2) indicate that all mice immunized with mutants corresponding to insertion of the HBc epitope at positions 140/141, 179/180, 266/267, 283/284, and 352/353 developed anti-HBc antibodies, as observed for mice immunized with HBc VLPs. In contrast, none of the four mice immunized with VLPs composed of L1 protein with the HBc78–83 epitope inserted at position 56/57 developed anti-HBc antibodies. Anti-HBc geometric mean titers of 84, 100, 237, 100, and 79 were observed with mutants 140/141, 179/180, 266/267, 283/284, and 352/353, respectively. A geometric mean titer greater than 3200 was observed in mice immunized with HBc VLPs.

Discussion

The aims of this study were to introduce a heterologous sequence into the HPV-16 L1 protein to characterize the conformational neutralizing epitopes of HPV-16 L1 VLPs, and the regions where a foreign epitope could be introduced at the surface of the VLPs. X-ray crystallographic analysis of HPV-16 L1 pentamers have identified loops at the surface of the structure (Chen et al., 2000). Because mutations

into loops have lower chances of affecting the tridimensional protein structure compared to mutations into α helices or β sheets, L1 loops appeared to be the most promising positions for insertion of an exogenous sequence into the L1 protein. The study focused on loops BC, DE, EF, FG, and HI, where residues involved in L1 B cell epitopes have been identified (Christensen et al., 1996; Ludmerer et al., 1996; McClements et al., 2001; Roden et al., 1997; White et al., 1999). An exogenous sequence, DPASRE, corresponding to the HBc epitope 78–83 was introduced at position 56/57, 140/141, 179/180, 266/267, 283/284, or 352/353 of the L1 protein. We evaluated the ability of the above L1 mutant proteins to form particles. All the chimeric L1 proteins produced self-assembled into VLPs of similar appearance to VLPs composed of wild-type L1 protein. However, larger VLPs were observed for the mutant with insertion in the BC loop and smaller VLPs for the two mutants with insertion in the FG loop. Thus, insertion of six amino acids into loops does not affect assembly of the HPV-16 L1 protein at the positions investigated, although other mutations within loops destabilize the structure (Kirnbauer et al., 1993; unpublished data).

Using murine immune sera obtained with wild-type HPV-16 L1 VLPs, the antigenicity of the chimeric VLPs composed of L1 proteins containing the HBc78-83 epitope at position 52/53, 140/141, 179/180, or 352/353 appeared similar to that of wild-type VLPs. In contrast, the antigenicity of the L1 mutant containing the HBc sequence at position 266/267 or 283/284 was reduced and suggested that L1 immunodominant epitopes are located within the FG loop. To determine whether epitopes recognized by anti-L1 MAbs (H16.V5, H16.E70, and H16.U4) were affected by insertion of a foreign sequence, we tested the reactivity of these antibodies to chimeric VLPs. The reactivity of H16.U4 MAb was similar with wild-type and mutant VLPs. In contrast, the reactivity of the other two monoclonal antibodies (H16.V5 and H16.E70) to HBc/HPV-16 VLPs was dramatically reduced when insertion into L1 protein was performed at position 266/267 or 283/284. These findings confirmed that H16.V5 and H16.E70 have overlapping binding sites, which are distinct from the H16.U4 epitope (White et al., 1999). Our findings also suggest that the H16.V5 and H16.E70 binding sites involve FG loop amino acids. They confirmed results recently published by Slupetzky et al. (2001) indicating that insertion between residues 286/287 and 281/287 affects H16.V5 and H16.E70 epitopes and by Christensen et al. (2001) indicating that H16.V5 antibody is able to bind hybrid VLPs H11:16-(266–297). Our findings are also in agreement with the observation that the H16.E70 epitope is lost or restored by substitutions at positions 266 and/or 282 (Roden et al., 1997; White et al., 1999). Residue 50 of the HPV-16 L1 protein has previously been shown to be important for H16.V5 and H16.E70 epitopes (White et al., 1999), suggesting that, in addition to the FG loop, the BC loop is part of the antibody binding site. Since our insertion at position 56/57 had no effect on the H16.V5 and H16.E70 epitopes, our findings could indicate that the BC loop is not involved in these epitopes and suggest that mutation at position 50 acts at a distance by distorting the VLP conformation, as also proposed by Christensen et al. (2001). This is in agreement with the fact that we observed that the F to L mutation at position 50 of the HPV-16 L1 gene of the Phil strain (Touzé et al., 1998), a strain identical to the 114K strain, abolished the capacity of the L1 protein to self-assemble into VLPs (unpublished data).

When injected into animals HPV VLPs induce high levels of antibodies directed at both linear (Christensen et al., 1996; Ludmerer et al., 1997) and conformational (Christensen et al., 1996; Ludmerer et al., 1996, 1997) epitopes. Evidence suggests that antibodies, such as H16.V5, H16.E70, and H16.U4 MAb, that are generated by conformational epitopes are neutralizing (Christensen et al., 1994a, 1994b; Giroglou et al., 2001; Roden et al., 1996, 1997; Rose et al., 1994; White et al., 1998, 1999). The epitope recognized by H16.V5 MAb has been suggested to be immunodominant since H16.V5 MAb is able almost completely to block HPV-16 VLP recognition of 75% of human HPV-16-positive sera (Wang et al., 1997). In order to investigate whether VLPs with reduced H16.V5 reactivity have a reduced capacity to induce neutralizing antibodies, mice were immunized with the different chimeric HBc/VLPs. The results indicate that the immunogenicity of the chimeric VLPs composed of L1 protein with a heterologous sequence inserted in loops BC, FG, or HI (position 56/57, 266/267, 283/284, or 352/353) were slightly less immunogenic than VLPs composed of wild-type L1 protein, mutant 266/267 being the least immunogenic capsid. However, immunization with chimeric VLPs induced neutralizing antibodies, with mutant 266/267 inducing the lowest neutralizing activity. This suggests that the FG loop is involved in the production of neutralizing antibodies. Interestingly, VLP mutants with the HBc epitope at position 283/284, but deficient in H16.V5 and H16.E70 epitopes, induced neutralizing antibodies at only half the level observed with wild-type VLPs, suggesting that the H16.V5 epitope is not the immunodominant neutralizing epitope but could be close to the immunodominant epitope. The H16.V5 MAb might therefore block HPV-16 VLP recognition of human HPV-16-positive sera by steric inherence. This also suggests the presence of other neutralizing epitopes within the L1 major capsid protein that could become immunodominant when H16.V5 and H16.E70 epitopes are lost, as in the situation described for epitopes on the HIV1 gp41 protein (Cleveland et al., 2000).

Our findings also contribute to the identification of positions within the L1 structure where foreign sequences can be introduced and expressed on the surface of VLPs. When inserted into loops DE, EF, FG, and HI (position 140/141, 179/180, 266/267, 283/284, or 352/353), the HBc epitope induced production of specific antibodies. These findings

Table 3
Sequence of oligonucleotides used for construction of HBc78-83/HPV16 L1 genes

Name	Sequence
INS5′ 56/57	AC**GACCCAGCGTCCCGGGAA**AATAACAAAATATTAGTTCC
INS5′ 56/57	TT**TTCCCGGGACGCTGGGTC**GTTAGGTTTTTTAATAGGAA
INS5′ 140/141	GT**GACCCAGCGTCCCGGGAA**GTGGATAATAGAGAATGTAT
INS3′ 140/141	AC**TTCCCGGGACGCTGGGTC**ACCTGCATTTGCTGCATAAT
INS5′ 179/180	CA**GACCCAGCGTCCCGGGAA**GTAAATCCAGGTGATTGTCC
INS3′ 179/180	AC**TTCCCGGGACGCTGGGTC**TGCAACATTGGTACATGGGG
INS5′ 266/267	CT**GACCCAGCGTCCCGGGAA**GTTGGTGAAAATGTACCAGA
INS3′ 266/267	AC**TTCCCGGGACGCTGGGTC**AGCACCAGCCCTATTAAATA
INS5′ 283/284	CT**GACCCAGCGTCCCGGGAA**GCAAATTTAGCCAGTTCAAA
INS3′ 283/284	GC**TTCCCGGGACGCTGGGTC**AGTAGACCCAGAGCCTTTAA
INS5′ 352/353	AA**GACCCAGCGTCCCGGGAA**ACTACATATAAAAATACTAA
INS34 352/353	GT**TTCCCGGGACGCTGGGTC**TTCTGAAGTAGATATGGCAG
HPV5′ L1 16	CC**AGATCT**ATGTCTCTTTGGCTGCCTAGTGAGGC
HPV3′ L1 16	CC**AGATCT**TTACAGCTTACGTTTTTTGCGTTTAG

Note. Bold and underlined sequences correspond to the HBc78-83 encoding sequence. Bold sequences are *Bgl*II restriction sites.

confirm those obtained by Christensen et al. (2001), who generated antibodies against HPV-16 sequences introduced into FG and HI loops of HPV11. This is also in agreement with the results of Chackerian et al. (1999) and Slupetzky et al. (2001), who reported that an immune response against a heterologous sequence could be obtained by insertion into the DE and HI loops of BPV-1, respectively. We found that insertion into the FG loop of HPV-16 at position 266/267 is the most effective for the induction of anti-HBc antibodies, suggesting the presence of the heterologous sequence on the VLP surface and in correlation with the fact that the HPV-immunodominant epitope is situated on this loop. However, the HBc reactivity could be explained by degradation or misfolding of the VLPs. The different chimeric VLP preparations were of similar appearance, mainly well-formed VLPs, when observed by electronmicroscopy, and the fact that no anti-HBc immune response was induced by L1-HBc 56/57 L1 VLPs suggests that the anti-HBc reactivity of the other chimeric VLPs was due to the presence of the HBc epitope on the surface of the VLPs rather than to their partial degradation or misfolding.

In conclusion, our findings indicate that chimeric L1 proteins obtained by insertion of a foreign sequence within the different loops retain VLP assembly properties. Our results also support the role of the FG surface loop as an HPV-16 neutralizing determinant and suggest that insertion of a foreign sequence into this loop disrupts the H16.V5 epitope and the overlapping H16.E70 epitope, but only reduces (and does not abolish) the capacity of chimeric VLPs to induce neutralizing antibodies. Our findings also define the regions where a foreign epitope could be introduced and exposed on the surface of VLPs and therefore provide a rational approach to designing better prophylactic HPV vaccines that can provide protection against multiple HPV types by inserting L1 or L2 epitopes in order to generate higher levels of cross-protection.

Materials and methods

Generation of HPV-16 L1 mutants

DNA-encoding chimeric L1 proteins were obtained by mutagenesis using a two-step PCR protocol. In the first step, one fragment was generated using the wild-type L1 DNA sequence as template and HPV5′L1 16 and INS3′ 56/57, INS3′ 140/141, INS3′ 179/180, INS3′ 266/267, INS3′ 283/284, and INS3′ 352/353 (Table 3) as primers for mutants L1 56/57, 140/141, 179/180, 266/267, 283/284, and 352/353, respectively. Another fragment was amplified using the wild-type L1 DNA sequence as template and HPV3′L1 16 and INS5′ 56/57, INS5′ 140/141, INS5′ 179/180, INS5′ 266/267, INS5′ 283/284, and INS5′ 352/353 (Table 3) as primers for mutants L1 56/57, 140/141, 179/180, 266/267, 283/284, and 352/353, respectively. The two fragments generated for each mutant contained the HBc78–83 encoding sequences in 3′ and 5′ and a *Bgl*II restriction site in 5′ and 3′, respectively. These fragments overlapped over their 3′ and 5′ sequences and were used in the second PCR step as DNA templates using HPV5′L1 16 and HPV3′L1 16 primers. The resulting DNA sequences had an HBc78–83-encoding sequence between L1 bases 168/169, 420/421, 537/538, 798/799, 849/850, and 1056/1057 and in 3′ and 5′ *Bgl*II restriction sites for the mutants L1 56/57, 140/141, 179/180, 266/267, 283/284, and 352/353, respectively. The wild-type L1 gene was amplified using DNA extracted from a cervical cancer patient from the Philippines (Touzé et al., 1998) as template and HPV5′ L1 16 and HPV3′ L1 16 as primers. These fragments contained a *Bgl*II restriction site in 3′ and 5′.

The HPV-16 L1 gene and chimeric HBc78–83/HPV-16 L1 genes were then cloned into the PCRII-TOPO vector (Invitrogen, Cergy Pontoise, France) after addition of A at PCR fragment extremities using *Taq* polymerase (Invitrogen) and were then sequenced with an ABI PRISM 377

automated sequencing system (Perkin–Elmer/Applied Biosystems, Courtaboeuf, France).

Generation of VLPs

HPV-16 L1 gene and chimeric HBc78–83/HPV-16 L1 genes obtained from the pCRII-TOPO vector by digestion with the *Bgl*II restriction enzyme were subcloned into the pFastBacDual plasmid (Invitrogen) linearized with a *Bam*HI restriction enzyme. Recombinant baculoviruses encoding HPV-16 L1 protein and chimeric HBc78–83—HPV-16 L1 proteins were generated using the Bac-to-Bac baculovirus expression system according to the manufacturer's instructions (Invitrogen).

Sf21 cells, maintained in Grace's insect medium supplemented with 10% fetal calf serum (FCS, Invitrogen), were infected with the different recombinant baculoviruses and incubated for 72 h at 27°C. Cells were harvested (500 *g* for 10 min), resuspended in phosphate-buffered saline (PBS) containing Nonidet P-40 (0.5%), pepstatin (10 μg/ml, Sigma Aldrich, Saint Quentin Fallavier, France), and leupeptin (1 μg/ml, Sigma Aldrich), and allowed to stand for 30 min at 4°C. Cell lysates were then centrifuged at 14,000 *g* for 15 min at 4°C. Pellets were resuspended in PBS containing pepstatin (10 μg/ml) and leupeptin (1 μg/ml) and sonicated by three 15-s burst at 60% maximal power (Vibra Cell; Bioblock Scientific, Strasbourg, France). Fractions were then loaded on the top of a preformed CsCl gradient and centrifuged at equilibrium in a Beckman SW28 rotor (22 h, 27,000 rpm, 4°C). Gradient fractions were analyzed for density by refractometry using an Abbé refractometer (Bioblock Scientific) and were tested for the presence of wild-type or chimeric L1 protein by electrophoresis in a 10% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) followed by Coomassie blue staining. CsCl fractions containing L1 protein were pooled, diluted in PBS, and pelleted in a Beckman SW 28 rotor (3 h, 28,000 rpm, 4°C). After centrifugation, VLPs were resuspended in 0.15 M NaCl and sonicated by one 5-s burst at 60% maximal power. Protein content was determined using the MicroBCA kit (Pierce, Touzard et Matignon, France) and assembly into VLPs verified by electron microscopy. For this purpose, purified VLP preparations were applied to carbon-coated grids, negatively stained with 1.5% uranyl acetate, and observed at ×50,000 nominal magnification using a JEOL 1010 electron microscope.

For generation and detection of anti-HBc antibodies, HBc VLPs were generated according to a previously described procedure (Touzé and Coursaget, 1998) with the exception that the full-length HBc gene was cloned and expressed.

Antibodies

Anti-HPV-16 L1 MAbs H16.V5, H16.E70, and H16.U4 were the kind gifts of N.D. Christensen (The Milton S. Hershey Medical Center, Hershey, PA, USA). CamVir-1 MAb directed against L1 epitope 204–210 (McLean et al., 1990) was obtained from Pharmingen (Newcastle, UK).

Anti-L1 VLP antisera were also obtained by subcutaneous immunization of four mice with each of the VLP preparations. Six- to 8-week-old female C57BL/6 mice (IFFA Credo, St. Germain l'Arbresle, France) received 5 μg of purified VLPs composed of wild-type or chimeric L1 protein in 0.15 M NaCl and 0.2% $AlOH_3$ as adjuvant. The immunization schedule consisted of one injection at days 0, 6, 14, and 28. Blood samples were obtained 11 days after the last injection. For the group immunized with mutant 352/353, one mouse died during the experiment and thus results were recorded in three animals.

Anti-HBc antiserum was obtained as previously described for anti-L1 VLP antiserum except that immunization was performed with purified HBc VLPs.

As for neutralizing antibodies against HPV-16, the serum samples were tested for antibodies against both anti-L1 and anti-HBc antibodies.

Detection of anti-L1 and anti-HBc antibodies by ELISA

Two hundred nanograms of VLPs, native or dissociated by treatment with 0.3 M Na_2CO_3, pH 10.6, and 0.01 M dithiotreitol (DTT) in PBS for 15 min at 37°C, was distributed in a 96-well plate (Nunc Maxisorp, Merck Eurolab, Strasbourg, France) and incubated at 4°C overnight. After two washing with PBS–Tween (0.1%), the wells were saturated with PBS supplemented with 1% FCS for 1 h at 37°C. Anti-L1 or anti-HBc antibodies diluted in PBS–Tween (0.1%)–FCS (1%) were then added to the wells and incubated for 1 h at 37°C. After four washes, peroxidase-conjugated goat antimouse Ig Fc (Sigma Aldrich) diluted 1:1000 in PBS–Tween (0.1%)–FCS (1%) was added to the plates and incubated for 1 h at 37°C. After four washes, 0.4 mg/ml of *o*-phenylenediamine and 0.03% hydrogen peroxide in 25 mM $(CH_2COOH)_2$ and 50 mM Na_2HPO_4 were added. After 30 min, the reaction was stopped with 4 N H_2SO_4 and absorbance was read at 490 nm. For data analysis, optical density (OD) values obtained in the absence of the first antibodies were subtracted from OD values of test samples.

All experiments were performed twice independently and yielded similar results.

Detection of neutralizing antibodies

Neutralization of the pseudovirions with anti-VLP antibodies was investigated by inhibition of gene transfer according to a previously described protocol (Bousarghin et al., 2002). Briefly, 10,000 SV40-transformed COS-7 kidney cells, cultured in complete Dulbecco's modified Eagle's medium (Invitrogen, DMEM supplemented with 10% FCS, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin) were seeded in 96-well plates. After 1 day at 37°C, cells

were washed twice with incomplete DMEM medium and resuspended by pipetting into 50 μl of incomplete DMEM medium. VLPs was mixed with pCMV Luc plasmid (Clontech, Ozyme, Montigny le Bretonneux, France) linearized by *Eco*RI digestion in 20 μl of 33 mM NaCl, pH 5, in a 10:1 weight ratio. After 30 min incubation at room temperature the pseudovirions obtained were then added to each test well. After 3 h at 37°C, 150 μl of complete DMEM was added to cells. Luciferase gene expression was measured by luminescence assay (Luciferase reporter gene assay with constant light signal, Roche Molecular Biochemical, Meylan, France). The luminescence was integrated over 10 s (Victor^2, Wallac, Perkin–Elmer, France) and results were expressed as counts per second (cps) per well. The quantity of DNA/VLP complexes was adjusted to give a luciferase activity of around 1500 cps corresponding to 10 ng of DNA per well. Thirty microliters of anti-VLP antibodies diluted from 1/50 to 1/6400 in incomplete DMEM medium was added to pseudovirions and incubated at 37°C for 30 min before addition to the cells. Cells were then incubated for 48 h at 37°C and luciferase gene expression was measured by luminescence assay. Neutralizing antibodies were considered to be present for inhibition greater than 80%. The experiments were performed in duplicate. Results were expressed as the mean geometric titers obtained for the four mice immunized with the same chimeric VLPs.

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